Flowcytometric Analysis of Tumor Associated Macrophages in Invasive Ductal Carcinoma of Breast

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ABSTRACT

Background: Invasive ductal carcinoma is the most common type of breast cancer in Iran. Impaired immune responses occur frequently in cancer patients, but the mechanisms of the induced immune defects remain poorly understood. It is believed that infiltrated immune cells, especially macrophages, may provide help for tumor cell growth and metastasis. **Objective:** To analyze the status of tumor associated macrophages (TAM) by immunophenotyping method. **Methods:** Twenty-three women suffering from breast cancer were examined; nineteen of them were confirmed histologically to have invasive ductal carcinoma. Tumor cell suspensions from biopsy specimens and peripheral blood mononuclear cells from patients and matched controls were processed for analysis by flow cytometry. **Results:** No significant changes in the percentages of intra-tumor leukocytes and macrophages in the different stages of tumor were observed. There were no significant differences in the percentages of leukocytes ($CD45^+$), monocytes ($CD45^+/CD14^+$) and activated monocytes ($CD14^+/HLA\text{-}DR^+$ and $CD14^+/CD16^+$) in the peripheral blood of patients and controls. **Conclusion:** The results of this study indicated that human breast cancer contain substantial, although variable numbers of macrophages, however, the activation status of these macrophages remain to be elucidated.

Keywords: Breast Cancer, Tumor Associated Macrophages, Tumor Immunity

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INTRODUCTION

Invasive ductal carcinoma is the most common type of breast cancer in Iran. Impaired immune responses occur frequently in cancer patients, but the mechanisms of the induced immune defects remain poorly understood (1). In many carcinomas, infiltrating macrophages are commonly found closely associated with tumor but little is known concerning the nature or significance of adherent molecules in these cellular interactions (2). In addition, the presence of immature dendritic cells within the tumor has been reported, whereas mature dendritic cells are located in peri-tumoral area (3).

In many solid tumors the abundance of tumor-associated macrophages (TAMs) is correlated with poor prognosis (4). Current evidence suggests that in established, progressively growing solid tumors, TAMs are reprogrammed to induce immune suppression of host defenses in situ, through release of specific cytokines, prostanoids and other humoral mediators. This disordered response, results in the inhibition of effective anti-cancer cell-mediated immune mechanisms. Concurrently, TAMs produce tumor growth promoting factors (5). Other studies have shown that macrophage expression urokinase (uPA) plays a key role in the degradation of tumor matrix and promotes tumor progression (6).

The summation of this complex interplay of biological factors results in progressive tumor growth and tumor cell dissemination. A better understanding of these complex interrelationships should form the basis of novel strategies to eradicate tumor cells in man and animals. Previous studies suggested the production of cytokines as a significant parameter in the regulation of tumor prognosis and indicated a defect in IL-12 production capability while generating higher amounts of IL-10 (7).

In this study we aimed to measure the percentage of macrophages infiltrated in tumor by flowcytometer, and estimate the activity of these cells in different stages of breast tumors.

MATERIAL AND METHODS

Patients. Twenty-three patients with breast cancer who underwent surgical procedure were studied in Cancer Institute of Imam-Khomeini hospital, Tehran. Patients with a history of chemotherapy and/or radiotherapy were excluded from study. The age of patients ranged from 26-70 yr (mean 45.4). Nineteen of the patients were diagnosed pathologically to have invasive ductal carcinoma (82.6%), two of the patients had invasive lobular carcinoma (8.7%), one patient had ductal carcinoma in situ, one patient had atypical modularly carcinoma and one patient had fibrocystic breast disease. Peripheral blood of nine cancer patients and 9 matched control women were collected in EDTA-coated tubes. The mononuclear cell fraction of blood samples were isolated by ficole and processed for immunostaining (see below). The patients and controls participated in this study after informed consent.

Preparation of Tumor Cell Suspension. Biopsies of human solid tumor were cut into small pieces with forceps and scalpel. The pieces were rinsed twice with phosphate buffer saline, then 5 ml of cocktail enzymes (0.05 mg/ml collagenase and 0.002 mg/ml DNAse) were added and the mixture was placed in 37^{o}C for 2-4 hrs. The suspension was passed through 150-micron stainless steel mesh. Cells were washed twice and labeled with monoclonal antibodies.

Immunophenotyping. The following fluorescent monoclonal antibodies (mAb) were used to identify leukocytes and TAM in both PBL and tumor cell suspensions: anti-CD45 for detection of leukocytes' anti-CD45/CD14 for detection of monocyte/macrophages and both anti-CD14/CD16 and anti-CD14/HLA-DR for detection of activated monocyte/macrophages (8). We established the reference immunophenotypic pattern using stan-

dard procedures. In this study 100µl of each blood sample was treated as follows; each sample was immunostained with 10µl of each mAbs directly conjugated with Fluorescein Isothiocyanate (FITC) or R-Phycoerythrin (RPE). Each sample was then fixed with paraformaldehyed and kept in 2-8 ^{o}C and dark for about 24 hrs (8).

Flowcytometric Analysis. Cell sample were measured on a coulter flowcytometer with serial filter configuration. The analysis was focused on the myeloid areas of the forward and side scatters. Double stained cells were analyzed using coulter software.

Statistical Analysis. One-way analysis of variation (ANOVA) and Kruskal-Wallis nonparametric test were employed using SPSS software.

RESULTS

Comparison of PBL monocytes between breast cancer patients and controls

Table 1 summarized the percentages of PBL leukocytes and monocytes in both patients and control group. Flowcytometric analysis was done in the gate of monocyte. Results indicated no significant differences in the percentage of leukocytes and monocytes compared to the control group.

The percentage of $CD16^+$ cells showed significant differences between two groups (P = 0.007) and the number of these cells showed a significant decrease in breast cancer patients.

Table1. Flowcytometric analysis of leukocytes and monocytes in peripheral blood of breast cancer patients and control group

CD Marker	Breast Cancer (n = 9)	Control (n = 9)	P value
$CD45^+$ [a]	88.5	92.5	0.2
$CD45^+/CD14^+$	2	3.1	0.44
$\frac{CD45^+/CD14^+}{CD45^+} \times 100$	2.7	3.4	0.44
$CD16^+$	19.7	54.2	0.007
$CD14^+/CD16^+$	0.4	0.9	0.1
$\frac{CD14^+/CD16^+}{CD45^+} \times 100$	0.4	1	0.1
$HLA\text{-}DR^+$	8.4	6.6	0.3
$D14^+/HLA\text{-}DR^+$	0.6	0.7	0.44
$\frac{D14^+/HLA\text{-}DR^+}{CD45^+} \times 100$	0.6	0.8	0.44

[a] values are shown in mean percentages and analysis was done in monocyte gate

Percentage of Tumor Associated Macrophages. In order to evaluate the percent of intratumor TAM, 23 patients were divided into two groups (IDC group and ILC+DCI+AMC group). The normal or benign tissues were considered as group 3. All the data was analyzed in the leukocyte gate. As shown in Table 2 the mean percentage of intratumor $CD14^+/HLA\text{-}DR^+$ cells were significantly (P = 0.03) higher in control group compared with breast cancer groups (group 1 and 2).

Correlation between TAM and Clinical Staging of Tumor. In order to assess the correlation between the percentage and activity of peripheral blood monocytes and TAM in different stages of tumor, patients having stages 1, 2 and 3 of the disease were evaluated. Results in table 3 indicated no significant differences between different stages of tumor. The $CD45^+$, $CD\ 14^+/CD16^+$ and $CD14^+/HLA\text{-}DR^+$ cells decreased remarkably in stage II of breast cancer

and in the cases of $CD45^+$ and $CD14^+/HLA\text{-}DR^+$ cells, this decrease was considerable but statistically insignificant (Table3).

Table 2. Imunostatus of tumor associated macrophages in breast cancer patients

Marker	Group 1 (n =19) IDC*	Group 2 (n = 4) ILC+DCI+AMC*	Group 3 (n = 3) Benign/control	P value
$CD45^+$	16.4	19.53	12.85	0.9
$CD45^+/CD14^+$	2.64	1.86	3.72	0.4
$\frac{CD45^+/CD14^+}{CD45^+} \times 100$	24.49	30.37	33.77	0.8
$CD16^+$	17.18	6.6	11.7	0.5
$CD14^+/CD16^+$	1.56	1.38	1.6	0.96
$\frac{CD14^+/CD16^+}{CD45^+} \times 100$	12.3	27.18	11.07	0.3
$HLA\text{-}DR^+$	14.71	11.18	19.5	0.4
$CD14^+/HLA\text{-}DR^+$	1.57	1.23	3.03	0.03
$\frac{D14^+/HLA\text{-}DR^+}{CD45^+} \times 100$	13.62	14.42	16.65	0.35

*IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma; DCI = Ductal carcinoma in situ; AMC = Atypical modularly carcinoma

In another analysis, the sizes of tumors correlated with the status of leukocytes and TAM. Results in Table 4 indicated no significant decrease in the total leukocyte and TAM count when the tumor size increased.

Table 3. Immunostatus of monocyte/macrophages in different stages of Invasive ductal carcinoma patients[a]

CD Marker	PBL cells				Intratumor cells			
	stages: I	II	III	P Value	stages: I	II	III	P Value
$CD45^+$	88.25	94.57	82.57	0.2	14	9.18	28.9	0.07
$CD45^+/CD14^+$	1.97	2.24	1.85	0.9	2.78	1.95	3.57	0.25
$CD14^+/CD16^+$	0.3	0.25	0.4	0.6	2.77	0.65	2.05	0.06
$CD14^+/HLA\text{-}DR^+$	0.63	0.43	0.63	0.8	2.33	1.23	1.5	0.35

[a] The minimum number of cases used in each stage of tumor was three

Table 4. Imunostatus of monocyte/macrophages in Invasive ductal carcinoma tumors with different sizes[a]

CD Marker	Intratumor Cells			
	<2cm	2-3cm	>3cm	P Value
$CD45^+$	19.6	9.8	19	0.6
$CD45^+/CD14^+$	2.8	2.8	2.3	0.9
$CD14^+/CD16^+$	2.2	1.2	0.9	0.3
$CD14^+/HLA\text{-}DR^+$	1.5	0.9	1.9	0.5

cm= centimeter

[a] The minimum number of cases used in each stage of tumor was three

DISCUSSION

The immune system is capable of responding to cancer as evidenced by systemic, regional, and intratumoral leukocyte activation. For individual patients there is no predictable relationship between leukocyte composition, or function, and the prognosis of the disease (9).

Immunohistological analysis of tissue sections and isolation of cells by mechanical desegregation and enzymatic digestion of excised tumors are the established methods used to study the cell composition of tumors (10). Previous published studies indicated that the number of macrophages is substantial but varies greatly among the different studied tumors (11,12). Macrophages also infiltrate metastatic lesions, although TAMs have been less extensively studied in secondary tumor deposits (13).

The Results of our study indicated that $CD16^+$ bearing cells significantly decrease in the PBL of breast cancer patients (Table 1). In the PBL population of human activated monocytes and natural killer (NK) cells express CD16 marker, therefore it is reasonable to assume a significant decrease of these two important cell populations in our patients.

We also evaluated the presence of macrophages within the invasive ductal carcinoma and other tumors of breast compared to fibrocystic benign tumor. The range of infiltrated $CD45^+$ cells was variable (2.7 and up to 61.7). The range of infiltrated $CD14^+$ cells was 0.7 and up to 4.5 which is consistent with previous results of van Rarenswaay Claasan et al. (11) and Toomey et al. (14).

This wide range of variation in cell infiltration is attributed to different stages of tumors and the rate of tumor growth. The activity of macrophages was studied by using $CD16^+$ and HLA-DR^+ markers. In stage II of breast cancer the CD16 and HLA-DR bearing macrophages decreased remarkably (Table 3) and this may confirm suppression of tumor infiltrating immune cells (5).

Solid tumors, both primary lesions and metastases, become infiltrated by large numbers of tumor-associated leukocytes. These are heterogeneous populations of cells consisting of various (and variable) subsets of T cells (helper, suppressor and cytotoxic), B cells, natural killer (NK) cells, and macrophages (11). However, the biological relevance and clinical significance of these different cellular infiltrates remains the subject of conflicting reports and continuing debate. In view of their normally defensive and beneficial role in vivo, leukocytes infiltrating tumor were originally believed to herald an immune response to the growing neoplasm. One of the first direct indications that killing of tumor cells by macrophages involved direct contact between them and tumor cells was provided by Bucana et al. (15). They produced morphological evidence on translocation of lysosomal organelles from cytotoxic macrophages into the cytoplasm of target cells. Electron microscopic observations have shown that destruction of tumor cells by activated macrophages is a non-phagocytic, lytic process (15). It is reported that the interaction between activated macrophages and the neoplastic cells involves intimate cellular binding, is temperature dependent and requires viable, metabolically active effectors cells (16). Recently co-cultivation of tumor cells with macrophages has been shown to enhance invasiveness of malignant cells in a TNF-alpha dependent manner (17). However, the spontaneous regression of an established tumor is a rare event, suggesting that in progressively growing tumors, the immunocompetence and anti-cancer effects of these cells within the tumor cell milieu may be compromised. Moreover, subpopulations of associated-associated leukocytes have been shown to be detrimental to the host and beneficial to progressive tumor growth (12,14).

It is possible that stimulation of the activity of macrophages can help us in increasing the binding of activated macrophages to tumor cells and selectively increase cytolysis of the tumor cells.

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